

12-Lipoxygenase is increased in glucose-stimulated mesangial cells and in experimental diabetic nephropathy

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Background. Arachidonic acid-derived 12-lipoxygenase (12-LO) products have potent growth and chemotactic properties. The present studies examined whether 12-LO and fibronectin are induced in cultured rat mesangial cells (MCs) exposed to high glucose and whether they are expressed in experimental diabetic nephropathy.

Methods. To determine the effect of high glucose on MC 12-LO mRNA and protein expression, rat MCs were incubated with RPMI medium containing 100 (NG) or 450 mg/dL glucose (HG). For animal studies, rats were injected with diluent (control) or streptozotocin. The latter were left untreated (DM) or treated with insulin (DM + I). At sacrifice after four months, GAPDH, 12-LO, and fibronectin mRNA were measured by competitive reverse transcription-polymerase chain reaction (RT-PCR) in microdissected glomeruli (G). Renal sections were semiquantitatively scored (0 to 4+) for diabetic changes and for 12-LO and fibronectin by immunohistochemistry.

Results. 12-LO mRNA expression in MC exposed to HG (12.71 ± 1.17 attm/ μ L) and DM G ($1.78 \pm 0.65 \times 10^{-3}$ attm/glomerulus) was significantly higher than those of MCs in NG media (6.71 ± 0.78 attm/ μ L) and control G ($0.34 \pm 0.12 \times 10^{-3}$ attm/glomerulus, $P < 0.005$), respectively. Western blot revealed a 1.7- and a 2.8-fold increase in MC and G 12-LO protein expression, respectively ($P < 0.05$). The immunohistochemistry score for G 12-LO and diabetic nephropathy score was significantly greater in DM and DM + I than controls. MC and G GAPDH mRNA remained unchanged.

Conclusions. In MCs exposed to HG and in diabetic rat glomeruli, increments in 12-LO mRNA and protein are associated with changes modeling diabetic nephropathy. These findings suggest a role for the 12-LO pathway in the pathogenesis of diabetic nephropathy.

Key words: glomerular microdissection, extracellular matrix, renal glomeruli, cell hypertrophy.

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The molecular and cellular mechanisms responsible for diabetic nephropathy remain incompletely resolved. While studies indicate involvement of hyperglycemia via the stimulation of growth factor-induced cellular hypertrophy [1, 2], increased production of extracellular matrix protein [3–6] and decreased production of matrix-degrading proteinases [7, 8], the underlying pathways mediating these processes have been explored less well.

The interaction of various growth factors with their cell surface receptors leads to signaling events and the activation of several phospholipases, which in turn can lead to the release of arachidonic acid [9, 10]. Arachidonic acid can be metabolized by at least three different pathways: the cyclooxygenase pathway to prostaglandins and thromboxane; the lipoxygenase pathway to lipoxygenase (LO) products such as 5-, 12-, and 15-hydroxy-eicosatetraenoic acids (HETEs) and leukotrienes; and the cytochrome P-450 epoxygenase pathway to epoxides and other products [11]. Exposure to or stimulated release of 12-lipoxygenase (12-LO) products plays a key role in cellular hypertrophic and mitogenic responses to growth factors [12–14]. In vascular smooth muscle cells, high glucose, angiotensin II, and platelet-derived growth factor-BB, all molecules that activate protein kinase C (PKC) and are purported to mediate diabetic nephropathy also stimulate a dose-dependent increases in 12-LO activity and expression [13, 14]. Indeed, the downstream effects of high-glucose concentrations and angiotensin II in vascular smooth muscle cells, including hypertrophy and extracellular matrix synthesis, are substantially abrogated by pharmacological inhibition of 12-LO [13], demonstrating the criticality of the lipoxygenase pathway in mediating the effects of these molecules. Exogenous provision of 12-HETE, a major product of the 12-LO pathway, leads to PKC translocation from the cytosol to membrane fractions and also activates specific PKC isoforms in adrenal glomerulosa cells, demonstrating the

capacity for PKC activation by lipoxygenase products [15]. In addition, 12-LO products, particularly under high-glucose conditions, stimulate extracellular matrix synthesis by vascular smooth muscle cells [16].

In kidney, the 5-LO pathway has demonstrated pathogenetic importance in experimental immune-mediated glomerular disease [17, 18], but it has not been evaluated in diabetic nephropathy. Evidence suggests that in glomeruli, 12- and 15-LO activity predominate, leading to the synthesis of 12- and 15-HETE [19, 20]. Rat tissues mainly express a leukocyte-type 12-LO [21], and Katoh et al have identified and partially sequenced a leukocyte-type 12-LO/15-LO from rat glomeruli [22]. Moreover, a high ambient glucose concentration has been shown to induce arachidonic acid release in mesangial cells (MCs), along with cyclooxygenase product synthesis, and PKC activation [23], but in the former study, the LO pathway was not assessed. Since MCs are similar in origin and behavior to vascular smooth muscle cells, this study was performed to evaluate whether 12-LO mRNA and protein are increased in glucose-stimulated MCs and in experimental diabetic nephropathy. Furthermore, studies were also performed to determine whether fibronectin production is also increased in association with the activation of the 12-LO pathway.

METHODS

Mesangial cell culture

Renal glomeruli were isolated from male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) weighing 250 to 300 g. Kidneys were removed. Glomeruli were isolated by differential sieving, and isolated glomeruli were incubated in collagenase and trypsin-ethylenediaminetetraacetic acid (EDTA) (GIBCO Laboratories, Bethesda, MA, USA) as described previously [24]. The identification of mesangial cells (MCs) was performed by their characteristic stellate appearance in culture and was confirmed by immunofluorescent microscopy for the presence of actin, myosin, and Thy-1 antigen and the absence of factor VIII and cytokeratin (Synbiotics, San Diego, CA, USA). MCs were maintained in RPMI 1640 (Mediatech Inc., Herndon, VA, USA) supplemented with L-glutamine, HEPES 7 mmol/L, and 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT, USA) and were incubated at 37°C in humidified 5% CO₂ in air.

To determine the effect of high glucose on MC 12-LO mRNA and protein expression, rat MCs were plated in 100 mm dishes for mRNA experiments and 60 mm dishes for Western blot analysis and were incubated with RPMI medium containing 100 mg/dL (NG) or 450 mg/dL (HG) glucose with 10% FCS, allowed to grow for five days to confluence, and serum starved with NG or HG medium containing 0.2% bovine serum albumin (BSA) and 0.4%

FCS for 24 hours. Thereafter, medium was changed to NG or HG medium with 0.2% BSA and was harvested after 24 hours.

Animals

All animal studies were conducted under an approved protocol. Thirty male Sprague-Dawley rats weighing 220 to 250 g were studied. Eight were injected with diluent [control (C)], and 22 were injected with 65 mg/kg streptozotocin intraperitoneally. Blood glucose levels were measured on the third day after streptozotocin injection to confirm the development of diabetes. Diabetic rats were then randomly assigned to two groups. One group, comprised of eight rats, was treated with 2 U/day of Humulin (DM + I; Ultralente; Eli Lilly Co., Indianapolis, IN, USA), and the remaining 14 diabetic rats were left untreated (DM). The rats were housed in a temperature-controlled room and were given free access to water and standard laboratory chow during the four-month study period.

Serum glucose and creatinine and 24-hour urinary albumin were measured monthly. Blood glucose was measured by glucometer, and serum creatinine was measured with a Creatinine Analyzer 2 (Beckman Instruments, Inc., Fullerton, CA, USA). Twenty-four-hour urinary albumin excretion was determined by ELISA (Nephra II; Exocell, Inc., Philadelphia, PA, USA). Body weights were measured weekly, and kidney weights were measured at the time of sacrifice.

Microdissection and total RNA extraction

Glomerular microdissection was performed as previously described [25, 26], with minor modifications. Fifty glomeruli from the renal cortex were microdissected at 4°C in a solution of vanadyl ribonucleoside complex and were rinsed and transferred to a tube with RNase inhibitor. Total RNA was extracted by the addition of 100 µL of RNA STAT-60 reagent (Tel-Test, Inc., Friendswood, TX, USA) to the glomeruli, followed by glomerular lysis by freezing and thawing three times. Another 700 µL of RNA STAT-60 reagent were added, and the mixture was vortexed and stored for five minutes at room temperature. Four hundred microliters of chloroform were added, and the mixture shaken vigorously for 30 seconds. After three minutes, the mixture was centrifuged at 12,000 × g for 15 minutes at 4°C, and the upper aqueous phase containing the extracted RNA was transferred to a new tube. RNA was precipitated from the aqueous phase by 400 µL isopropanol and pelleted with centrifugation at 12,000 × g for 30 minutes at 4°C. The RNA precipitate was washed with 70% ice-cold ethanol, dried using Speed Vac, and dissolved in DEPC-treated distilled water. RNA from tubulointerstitial tissue and MCs was extracted similarly. Glomerular, tubulointerstitial,

and MC RNA yield and quality were assessed based on spectrophotometric measures at 260 and 280 OD.

Reverse transcription

First-strand cDNA was made by utilizing a Boehringer Mannheim cDNA synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany). Total RNA from 50 glomeruli was reversed transcribed using 10 μ mol/L random hexanucleotide primer, 1 mmol/L dNTP, 8 mmol/L $MgCl_2$, 30 mmol/L KCl, 50 mmol/L Tris-HCl, pH 8.5, 0.2 mmol/L dithiothreitol, 25 U RNase inhibitor, and 40 U AMV reverse transcriptase. The mixture was incubated at 30°C for 10 minutes and 42°C for one hour, followed by inactivation of enzyme at 99°C for five minutes. Tubulointerstitial RNA, aliquoted in amounts equivalent to that of the glomerular samples, and 2 μ g of MC RNA from each plate were similarly reverse transcribed.

Quantitative competitive polymerase chain reaction

Competitor cDNAs were used as internal standards in quantitative polymerase chain reaction (PCR). Each competitor was designed to contain the same base pair sequence as the target cDNA that would allow efficient priming, but had a portion deleted so that the competitor PCR-generated fragment could be easily distinguished electrophoretically by size. Thus, to quantitate the rat GAPDH, leukocyte-type 12-LO [27], and fibronectin mRNA content of each sample, a competitive PCR technique was applied. The primers used for rat GAPDH, 12-LO, and fibronectin amplification were as follows: GAPDH sense, 5'-GACAAGATGGTGAAGGTCGG-3', antisense 5'-CATGGACTGTGGTCATGAGC-3'; 12-LO sense 5'-TGGGGCAACTGGAAGG-3', antisense 5'-AGAGCGCTTCAGCACCAT-3'; and fibronectin sense 5'-GCAAGCCTGAACCTGAAGAGACC-3', antisense 5'-CCTGGTGTCTCCTGATCATTGCATC-3'. The competitive PCR for 12-LO of MC was performed using nested PCR, and the sense and antisense primers for the first PCR were 5'-GGGCAGAAGCATCCTGAGCC-3' and 5'-GGAGTAGACCCAGTTTTGGG-3', respectively. cDNA from one tenth of a glomerulus or 0.5 ng RNA of MC per reaction tube was used for GAPDH and fibronectin and one half of a glomerulus or 25 ng RNA of MC for 12-LO. PCR was performed using the test or wild-type cDNA, Taq-Gold polymerase (1 U for GAPDH and fibronectin; 1.5 U for 12-LO), 20 μ mol/L dNTP, sense and antisense primers (20 pmol for GAPDH and fibronectin; 30 pmol for 12-LO), and serial dilutions of competitor in a volume of 50 μ L containing 1 \times PCR buffer. The PCR conditions were as follows: (1) GAPDH, 35 cycles, denaturation at 95°C for one minute, annealing at 62°C for one minute, and extension at 72°C for one minute; (2) fibronectin, 36 cycles, denaturation at 95°C for one minute, annealing at 65°C for one minute, and extension at 72°C for one

minute; and (3) 12-LO, 42 cycles, denaturation at 95°C for one minute, annealing at 60°C for one minute, and extension at 72°C for one minute. For competitive PCR of MC 12-LO, the first PCR was done by 35 cycles, and the nested PCR was done by 32 cycles with 0.1 μ L of the first PCR product under the same conditions as described previously in this article. Initial heating at 95°C for nine minutes and final extension at 72°C for seven minutes were performed for all PCRs. The reverse transcription-PCR (RT-PCR) products were separated by electrophoresis, the band densities were analyzed by laser densitometry (Helena Laboratories, Beaumont, TX, USA). The values log transformed, and a log-linear regression analysis was performed against the competitor concentration for each PCR tube. The quantity of cDNA in the test sample was defined as the amount at which the competitor and wild-type optical density bands were equal.

Western blot analysis

Rat glomeruli isolated using sieves and MCs harvested from plates were lysed in sodium dodecyl sulfate (SDS) sample buffer [2% SDS, 10 mmol/L Tris-HCl, pH 6.8, 10% (vol/vol) glycerol]. Lysate was centrifuged at 10,000 \times g for 10 minutes at 4°C, and the supernatant was stored at -70°C until all rats were sacrificed. Protein concentrations were determined with a Bio-Rad kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Aliquots of 50 μ g protein were treated with Laemmli sample buffer, then heated at 100°C for five minutes, and electrophoresed 50 μ g/lane in an 8% acrylamide denaturing SDS-polyacrylamide gel. Proteins were transferred to Hybond-ECL membrane (Amersham Life Science, Inc., Arlington Heights, IL, USA) using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA, USA). The membrane was incubated in blocking buffer A [1 \times phosphate-buffered saline (PBS), 0.1% Tween-20, and 8% nonfat milk] for one hour at room temperature and then incubated overnight at 4°C with a polyclonal antibody raised in rabbits to a specific leukocyte 12-LO peptide with sequence homology to amino acids 39 to 55 of porcine leukocyte 12-LO [14, 28]. The membrane was washed once for 15 minutes and twice for five minutes in 1 \times PBS with 0.1% Tween-20 and incubated in buffer A with horseradish peroxidase-linked sheep anti-mouse IgG (Amersham) at 1:1000 dilution. The washes were repeated, and the membrane developed with chemiluminescent agent (ECL; Amersham Life Science, Inc.).

Pathology

Coronal slices of kidney for routine light microscopy were placed into alcoholic Bouin's solution and were processed in the standard fashion, and sections were stained with periodic acid-Schiff. Slices of kidney for immunohistochemical staining were fixed in 10% neu-

tral-buffered formalin and processed in the standard manner, and 5 micron sections were utilized. Slides were deparaffinized, hydrated in ethyl alcohol, and washed in tap water. Antigen retrieval was carried out in 10 mmol/L sodium citrate buffer for 20 minutes using a Black and Decker vegetable steamer. For 12-LO staining, a polyclonal rabbit antiporcine 12-LO antibody (Dako, Carpinteria, CA, USA) diluted 1:300 with 2% casein in BSA was applied for overnight incubation at room temperature. After washing, the secondary swine anti-rabbit antibody was added for 20 minutes. Slides were washed and then incubated with a tertiary rabbit-PAP complex for 20 minutes. Diaminobenzidine was added for seven minutes, and slides were counterstained with hematoxylin. For fibronectin staining, the primary monoclonal mouse anti-Ec fibronectin antibody (Chemicon, Temecula, CA, USA) was diluted 1:100 with ChemMate antibody dilution buffer (Vantana Medical Systems, Tuscon, AZ, USA) and added for overnight incubation at 4°C. The remainder of the staining procedure was performed with reagents from and as specified in the ChemMate kit (Vantana Medical Systems, Tuscon, AZ, USA).

Animals were given a diabetic nephropathy score based on a 0 to 4+ semiquantitative score of glomerular changes, including mesangial widening, segmental glomerulosclerosis, insudative lesions, and capsular drops; all glomeruli in each section were examined. A semiquantitative score of 0 to 4+ was used for assessing 12-LO immunohistochemical staining separately within glomeruli assessing all glomeruli in the sections and in the tubulointerstitium by evaluating 20 consecutive fields using a $\times 20$ objective.

Statistical analysis

All values are expressed as the mean \pm SEM. Statistical analysis was performed using the statistical package SPSS for Windows Version 7.51 (SPSS, Inc., Chicago, IL, USA). Results were analyzed using Student *t* test or Kruskal–Wallis nonparametric test for multiple comparisons. If there was a significant difference by Kruskal–Wallis test, it was further confirmed by Mann–Whitney *U* test.

RESULTS

Mesangial cell culture studies

Quantitative competitive RT-PCR. The 12-LO mRNA of MCs exposed to HG (12.71 ± 1.17 attm/ μ L) was significantly higher than that of MCs in NG media (6.71 ± 0.78 attm/ μ L, $P < 0.005$). Fibronectin mRNA was also significantly higher in HG than NG cells (3.51 ± 0.35 vs. 2.21 ± 0.10 attm/ng RNA, $P < 0.005$). On the other hand, there was no significant difference in the amount of GAPDH mRNA between the two groups (NG, 3.76 ± 0.29 attm/ng RNA; HG, 3.41 ± 0.20 attm/ng RNA).

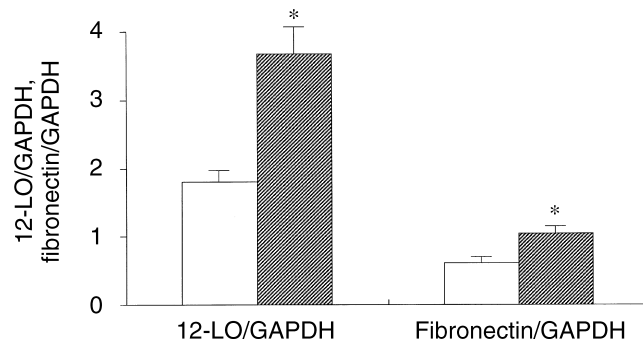


Fig. 1. Rat mesangial cell (MC) 12 lipoxigenase (12-LO)/GAPDH mRNA and fibronectin/GAPDH mRNA ratios after incubation in normal (□) or high (▨) glucose media. * $P < 0.005$ vs. NG.

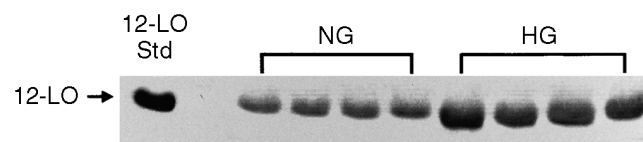


Fig. 2. 12-Lipoxygenase (12-LO) protein expression in normal (NG) and high (HG) glucose mesangial cells (MC). There was a 2.4-fold increase in 12-LO protein expression in HG compared with NG MCs assessed by densitometry ($P < 0.05$).

After correcting the amount of MC 12-LO and fibronectin mRNA for the amount of GAPDH mRNA, the differences between the two groups remained significant (Fig. 1).

12-LO protein expression in mesangial cells exposed to HG. 12-LO protein expression was significantly higher in HG than NG MC. There was a 2.4-fold increase in 12-LO protein expression in HG compared with NG cells assessed by densitometry ($P < 0.05$; Fig. 2).

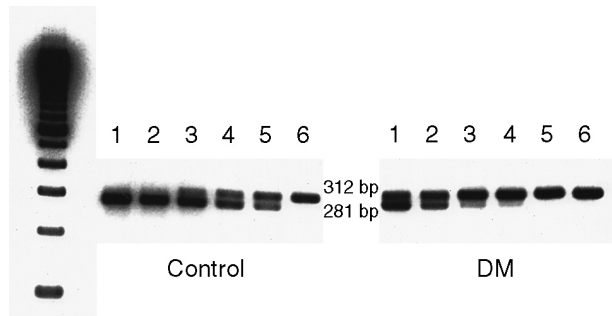
Animal studies

Animal data. The mean blood glucose levels of C, DM + I, and DM rats were 102.4 ± 1.8 , 294.2 ± 8.9 , and 415.0 ± 9.2 mg/dL, respectively ($P < 0.01$). Compared with the C group (4.59 ± 1.42 mg/day), 24-hour urinary albumin excretion was significantly greater in the DM + I (17.84 ± 5.79 mg/day, $P = 0.05$) and the DM (22.08 ± 4.94 mg/day, $P < 0.01$) groups at four months. All animals gained weight over the four-month experiment, but weight gain was highest in C rats, intermediate in DM + I, and least in DM ($P < 0.01$). The mean body weight after four months was 631.9 ± 15.1 g in the C, 495.6 ± 14.0 g in the DM + I, and 368.9 ± 9.5 g in the DM group. Kidney weight was measured in 16 rats at the time of sacrifice. The ratio of kidney weight to body weight in DM rats (0.87 ± 0.04) was significantly higher than C (0.36 ± 0.01) and DM + I rats (0.54 ± 0.04 , $P < 0.01$; Table 1).

Quantitative competitive RT-PCR. Glomerular 12-LO

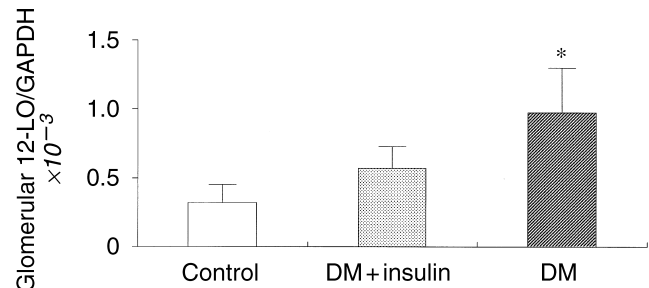
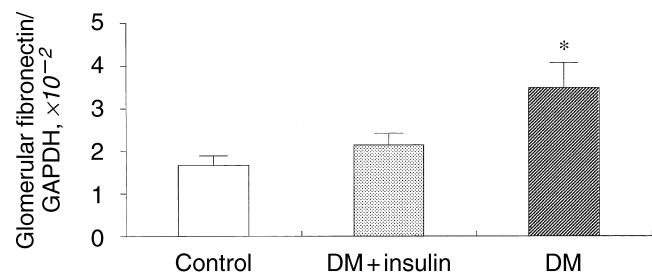
Table 1. Body weight, food intakes, kidney weight/body weight, blood glucose, and 24-hour urinary albumin excretion of the three groups

	Control (N = 8)	DM + I (N = 8)	DM (N = 14)
Body weight after 4 months g	631.9 ± 15.1	495.6 ± 14.0 ^a	368.9 ± 9.5 ^{a,b}
Blood glucose mg/dL	102.4 ± 5.0	294.2 ± 25.0 ^a	415.0 ± 34.3 ^{a,b}
24-hour urinary albumin excretion mg/day	4.59 ± 1.42	17.84 ± 5.79 ^c	22.08 ± 4.94 ^a
Kidney weight/body weight	0.36 ± 0.01 (N = 4)	0.54 ± 0.04 (N = 4)	0.87 ± 0.04 ^{a,b} (N = 8)

^a *P* < 0.01 vs. control^b *P* < 0.01 vs. insulin-treated diabetes (DM + I)^c *P* = 0.05 vs. control**Fig. 3.** A representative glomerular 12-LO RT-PCR electrophoresis of one control and one diabetic rat. Glomeruli were microdissected, and competitive RT-PCR was performed at four months after injection of diluent or streptozotocin, respectively. Each lane represents a competitive RT-PCR reaction (42 cycles) with a fixed amount of 12-LO wild-type cDNA (312 bp) from the equivalent of 0.5 glomeruli and a variable amount of 12-LO competitor cDNA (281 bp) as follows: (lane 1) 5×10^{-3} attm/ μ L, (lane 2) 10^{-3} attm/ μ L, (lane 3) 5×10^{-4} attm/ μ L, (lane 4) 10^{-4} attm/ μ L, (lane 5) 5×10^{-5} attm/ μ L, and (lane 6) 10^{-5} attm/ μ L.

mRNA was significantly higher (over 4-fold) in the DM group ($1.78 \pm 0.65 \times 10^{-3}$ attm/glomerulus) than the C group ($0.34 \pm 0.12 \times 10^{-3}$ attm/glomerulus, *P* < 0.005), while the amount in the DM + I was intermediate ($1.28 \pm 0.50 \times 10^{-3}$ attm/glomerulus; Fig. 3). Glomerular fibronectin mRNA was also significantly higher in DM than C rats ($5.65 \pm 0.81 \times 10^{-2}$ vs. $2.50 \pm 0.78 \times 10^{-2}$ attm/glomerulus, *P* < 0.01), and that in DM + I was intermediate ($4.29 \pm 0.71 \times 10^{-2}$ attm/glomerulus). There was no significant difference in the amount of glomerular GAPDH mRNA among the three groups (C, 1.38 ± 0.28 attm/glomerulus; DM + I, 2.36 ± 0.53 attm/glomerulus; and DM, 2.04 ± 0.33 attm/glomerulus). Even when corrected for GAPDH mRNA, glomerular 12-LO and fibronectin mRNA remained significantly higher in DM compared with C (12-LO/GAPDH, $0.99 \pm 0.31 \times 10^{-3}$ vs. $0.30 \pm 0.13 \times 10^{-3}$, DM vs. C, *P* < 0.01; fibronectin/GAPDH, $3.44 \pm 0.60 \times 10^{-2}$ vs. $1.67 \pm 0.19 \times 10^{-2}$, DM vs. C, *P* < 0.05; Figs. 4 and 5).

In contrast to the results in the glomerulus, tubulointerstitial 12-LO, fibronectin, and GAPDH mRNA levels did not differ among the three groups: (1) 12-LO: C,

**Fig. 4.** Glomerular 12-LO/GAPDH mRNA ratios of the three groups. The amount of glomerular 12-LO mRNA was corrected by the amount of glomerular GAPDH mRNA. There was a 3.3-fold increase in glomerular 12-LO mRNA expression in untreated diabetic rats (*N* = 14) compared with control rats (*N* = 8), while insulin-treated rats (*N* = 8) were intermediate. **P* < 0.01 vs. control.**Fig. 5.** Glomerular fibronectin/GAPDH mRNA ratios of the three groups. The amount of glomerular fibronectin mRNA was corrected by the amount of glomerular GAPDH mRNA. There was a 2.1-fold increase in glomerular fibronectin mRNA levels in untreated diabetic rats (*N* = 14) compared with control (*N* = 8), while insulin-treated rats (*N* = 8) were intermediate. **P* < 0.01 vs. control.

$4.51 \pm 2.17 \times 10^{-2}$ attm/ μ L; DM + I, $2.09 \pm 0.52 \times 10^{-2}$ attm/ μ L; and DM, $3.45 \pm 1.88 \times 10^{-2}$ attm/ μ L; (2) fibronectin: C, $2.05 \pm 0.87 \times 10^{-3}$ attm/ μ L; DM + I, $1.02 \pm 0.26 \times 10^{-3}$ attm/ μ L; and DM, $2.06 \pm 0.64 \times 10^{-3}$ attm/ μ L; and (3) GAPDH: C, 2.88 ± 0.82 attm/ μ L; DM + I, 1.89 ± 0.22 attm/ μ L; and DM, 2.59 ± 0.50 attm/ μ L. After correcting the amount of tubulointerstitial 12-LO and fibronectin mRNA for the control GAPDH mRNA, the values in the three groups were nearly identical (data not shown).

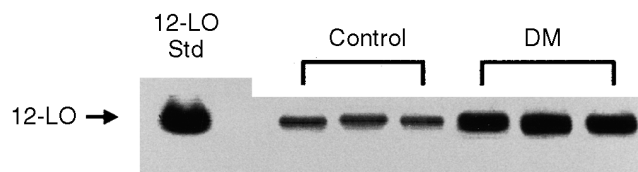


Fig. 6. A representative 12-LO Western blot of three control and three untreated diabetic rats. Densitometric quantitation revealed that there was a 2.8-fold increase in 12-LO protein expression in untreated diabetic rats compared with control rats ($P < 0.05$).

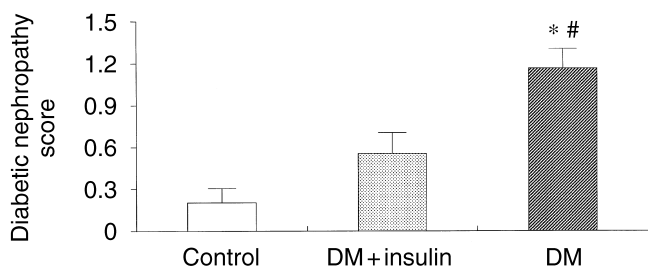


Fig. 8. Diabetic nephropathy score of the three groups. The score was determined semiquantitatively based on glomerular changes including mesangial widening, segmental glomerulosclerosis, insudative lesions, and capsular drops assessed by light microscopy. The score was significantly higher in the untreated diabetic group than the control and insulin-treated groups. * $P < 0.001$ vs. control; # $P < 0.05$ vs. insulin-treated diabetic group.

12-Lipoxygenase protein expression in diabetic kidney

Figure 6 is a representative Western blot showing expression of glomerular 12-LO protein in C and DM rats. 12-LO protein expression was higher in DM than C rats. Densitometric quantitation revealed that there was a 2.8-fold increase in 12-LO protein expression in DM compared with C rats ($P < 0.05$).

Pathology

The DM group had diffuse expansion of mesangial regions with occasional glomerular segmental sclerosis and insudates, and infrequent capsular drops resulting in a diabetic nephropathy score (1.17 ± 0.13) that was significantly higher than the C (0.21 ± 0.10 , $P < 0.001$) and DM + I (0.56 ± 0.15 , $P < 0.05$) groups (Figs. 7 and 8). Immunohistochemical staining for glomerular 12-LO confirmed the mRNA and Western blot findings. Glomerular 12-LO appeared to be both within mesangial and visceral epithelial cells and by semiquantitative scoring was significantly higher in DM (0.79 ± 0.11) and DM + I rats (0.53 ± 0.03) compared with C rats (0.29 ± 0.14 , $P < 0.05$; Figs. 7 and 9). There was patchy staining of tubular epithelial cells within proximal and distal tubules that did not reach statistical significance (C, 0.61 ± 0.24 ; DM + I, 0.72 ± 0.13 ; and DM, 0.90 ± 0.16). Transitional epithelium in the renal pelvis stained strongly for 12-LO in all three groups (data not shown). Fibronectin Ec

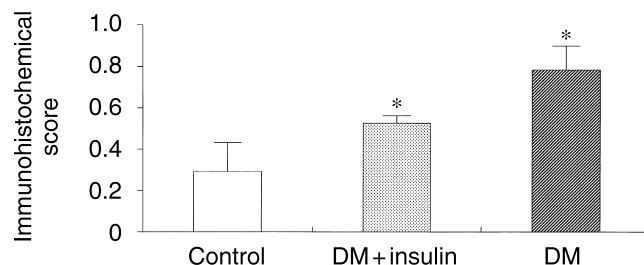


Fig. 9. Immunohistochemical score for glomerular 12-LO. The score was assessed semiquantitatively by the distribution and intensity of immunohistochemical staining. The score was significantly higher in diabetic rats (both untreated and insulin-treated) than control rats. * $P < 0.05$ vs. control.

staining was performed in DM and C groups only. C kidneys had no discernible fibronectin staining within glomeruli, while the DM animals had heavy staining within mesangial regions (Fig. 10), confirming the mRNA findings.

DISCUSSION

The 12-LO pathway has been shown to play a key role in the actions of effector molecules such as glucose, angiotensin II, and platelet-derived growth factor in vascular smooth muscle cells [13, 14, 16]. Our study demonstrates for the first time, to our knowledge, that glucose stimulates MC 12-LO mRNA and protein synthesis and that glomerular 12-LO mRNA and protein expression are increased in experimental diabetic nephropathy, suggesting a potential role of this pathway in the mediation of experimental diabetic nephropathy.

Mesangial cells in vitro predominantly synthesize 12-LO [19], and to a lesser extent, 15- or 5-LO products (abstract; Imai et al, *J Am Soc Nephrol* 1:751, 1990). In addition, urinary 12-HETE excretion is markedly increased in diabetic patients with or without microalbuminuria compared with healthy controls, suggesting a renal origin [30]. Thromboxane A_2 and 5-LO products originating from infiltrating inflammatory cells have been shown to be increased in studies of experimental glomerulonephritis and to mediate the decrements in glomerular filtration rate and renal blood flow which accompany MC immune injury [17, 18, 31, 32]. However, in contrast to immune-mediated glomerular disease, there is little cellular infiltration in diabetic nephropathy. Therefore, this study focused on 12-LO rather than other LOs. The ability of high-glucose medium concentration to stimulate MC 12-LO delineates at least one mechanism whereby the diabetic milieu might induce glomerular 12-LO in vivo. Thus, the data support the hypothesis that glomerular 12-LO may contribute to the development of diabetic nephropathy.

Numerous in vitro studies in nonrenal cells suggest

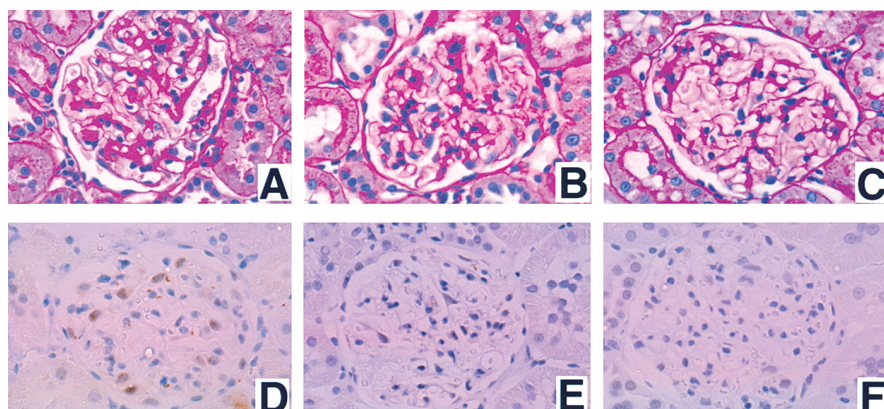


Fig. 7. Glomeruli from the untreated diabetic group (A and D) and the insulin-treated group (B and E). Mesangial regions in the untreated diabetic group are expanded with period acid-Schiff-positive material without hypercellularity (A). There are 12-LO-positive cells in mesangial regions and along capillary walls (D). There is mild mesangial expansion in the insulin-treated group (B), that is less than in the untreated diabetic group. There is weak staining for 12-LO (E). (C and F) Control group glomeruli. Mesangial regions have the normal width (C). There is no staining of any cells for 12-LO (F). (A–C) Periodic acid-Schiff $\times 260$. (D–F) 12-LO $\times 260$.

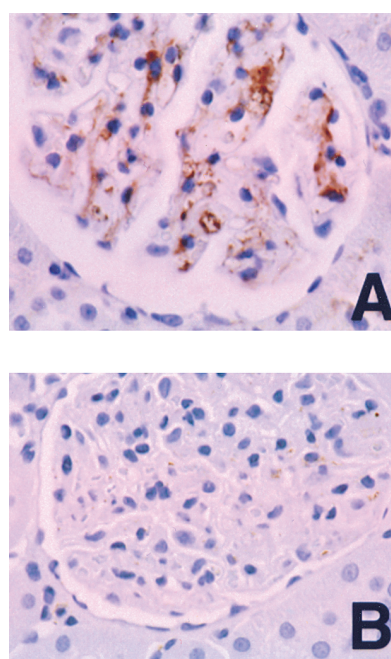


Fig. 10. Glomeruli stained for fibronectin-Ec. (A) Untreated diabetic group showing staining within mesangial regions. (B) Control group without mesangial staining ($\times 300$).

potential pathways by which 12-LO activation might participate in the pathogenesis of this disease. These demonstrate the role of the 12-LO pathway in regulating key signal transducing kinases and the role of 12-LO activation in stimulating fibronectin synthesis. Specifically, the capacity of the 12-LO product 12-HETE to activate PKC is demonstrated in experiments in adrenal glomerulosa cells, where exogenous provision of 12-HETE led to PKC translocation from the cytosol to membrane fraction [15]. PKC- γ activation by 12-HETE has also been demonstrated in brain cell culture [33]. LO metabolites of arachidonic acid have been reported to mediate tumor necrosis factor- α -induced protooncogene *c-fos* expression [34] and to increase *ras* activity [35]. Arachidonic

acid and its metabolites stimulated mitogen-activated protein kinase (MAP-kinase) activity in vascular smooth muscle cells [36] and 12-HETE led to sustained activation of MAP-kinases including *c-Jun* N-amino terminal kinase (JNK) [37, 38]. LO activation can directly lead to oxidant stress by the generation of superoxide [39], and this may mediate cell injury/nephropathy since oxidant stress plays a key role in diabetic complications [40]. 12-HETE, angiotensin II, and a high ambient glucose concentration stimulate fibronectin synthesis in vascular smooth muscle cells [13, 16]. Finally, LO inhibitors could block angiotensin II-induced vascular smooth muscle cell hypertrophy [16]. Thus, the 12-LO pathway could potentially mediate the glomerular changes of diabetic nephropathy through numerous mechanistic pathways.

In contrast to the results with glomerular samples, there was no difference in tubulointerstitial 12-LO, fibronectin, or GAPDH mRNA, and the immunohistochemical staining for 12-LO and fibronectin was similar among the three experimental groups. By immunohistochemistry, 12-LO protein showed focal and patchy distribution throughout the tubulointerstitium. There was relatively strong staining of 12-LO in the deep medulla and pelvis, a feature that was consistent in all three groups. For RNA extraction and RT-PCR of the tubulointerstitium, the studied renal tissue included both cortex and deep medulla. The heavy 12-LO expression in the medulla, as demonstrated by immunohistochemistry, may have obscured more subtle localized tubulointerstitial changes, thus explaining the discordance between the tubulointerstitial findings and the results in glomerular samples. It is known that glomerular MCs and tubular epithelial cells exhibit numerous similar responses under high-glucose conditions. For example, high glucose increases TGF- $\beta 1$ mRNA and protein in both cultured MCs and proximal tubular cells [41]. High glucose also increases decorin mRNA expression in cultured mouse mesangial and tubular epithelial cells [42]. However, 12-LO in tubular epithelial cells has not been studied,

and it is equally possible that activation in tubular epithelial cells may differ from that in MCs. In vitro studies of 12-LO using tubular epithelial cells will be necessary to clarify this question.

In rabbit tubular epithelial cells, arachidonic acid has been shown to activate JNK by stimulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and superoxide generation, independent of eicosanoid biosynthesis [43]. Recently, there was also a report that in interleukin-1 (IL-1)-stimulated MCs, only free arachidonic acid and its precursor linoleic acid—but not other fatty acids—stimulated JNK1/stress-activated protein kinase (SAPK), and their effects were independent of cyclooxygenase, lipoxygenase, and P-450 epoxigenase pathway [44]. Both reports focused only on JNK, but there are numerous signal transduction pathways besides those involving JNK that could mediate the pathogenesis of diabetic glomerulopathy. Because the former study was performed using tubular epithelial cells, there is a possibility that tubular cells may respond differently from glomerular cells. The latter study proposed a specific activator role for arachidonic acid to initial cellular activation by IL-1. Since numerous growth factors and cytokines besides IL-1 activate phospholipases, such as platelet-derived growth factor [45, 46] and epidermal growth factor [47, 48], the significance of these studies may be much wider than anticipated in implicating lipid mediators in the signal transduction pathways of a variety of cellular stimuli.

In summary, our study demonstrates that glucose per se stimulates MC 12-LO mRNA and protein synthesis, and that glomerular 12-LO mRNA and protein are increased in streptozotocin-induced experimental diabetic nephropathy. Taken together with previously published data in vascular smooth muscle cells, these results implicate the 12-LO pathway as a potential mediator of diabetic nephropathy and suggest new therapeutic strategies based on 12-LO inhibition.

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